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A histidine accepting tRNA-like fold at the 3′-end of satellite tobacco mosaic virus RNA

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ABSTRACT

A model of secondary structure is proposed for the 3′-terminal sequence of the satellite tobacco mosaic virus (STMV) RNA on the basis of phylogenetic comparisons with tobacco mosaic virus (TMV) genomic RNA. Sequence homologies and compensatory base changes found between the two related viral RNAs imply that the 3′-end of STMV RNA folds into a tRNA-like domain similar to that found in the TMV RNA. Accordingly, functional assays showed that STMV RNA can be aminoacylated *in vitro* with histidine by yeast histidyl-tRNA synthetase to plateaus reaching 30%. Histidylation properties of STMV RNA were compared to those of TMV RNA and of a canonical yeast $tRNA^{His}$ transcript which both are chargeable to nearly 100% plateau levels. Kinetic data indicate an excellent catalytic efficiency of STMV RNA charging expressed as V_{max}/K_m ratio, quasi-equivalent to that of TMV RNA, and only 17-fold reduced as compared to that of the yeast $tRNA^{His}$ transcript. Biological implications of the structural mimicry between the tRNA-like regions of TMV and STMV RNAs are discussed in the light of the relationships of a satellite virus with its helper virus. This is the first report on a chargeable tRNA-like structure at the 3′-end of a satellite virus RNA.

INTRODUCTION

Satellite tobacco mosaic virus (STMV) (1–4) is a T=1 icosahedral virus whose 60 identical capsid proteins of M_r 17,500 encapsidate a single stranded genome having 1059 bases of known sequence (5). STMV can replicate in its host, generally tobacco, only when the plant is co-infected with the helper tobacco mosaic virus (TMV), a rod shaped virus upon which it is dependent (4, 6). The STMV RNA does not show appreciable sequence homology with TMV RNA, in terms of either genome or coat protein amino acid sequences, except for the 3′-terminal 250 last nucleotides that exhibit 65% homology with the corresponding sequence of TMV RNA (5). The RNA of STMV codes only for its coat protein plus one other small protein of M_r 6,700 whose function, if any, is unknown (2, 4, 5).

The three-dimensional structure of STMV has been determined by X-ray diffraction analysis to high resolution (7, 8). An unique feature of this analysis was that segments of double helical RNA, each at least seven base-pairs in length, and arising from secondary structural elements in the RNA, could be observed associated on the interior with each pair of coat protein subunits making up the capsid. Altogether, nearly 45% of the entire RNA could be accounted for by these helical segments. One could conclude from this that the RNA inside the capsid must assume both secondary and tertiary structures consistent with the icosahedral distribution of the helical RNA segments on the interior of the virus. Because so much of the viral RNA structure was visible in this X-ray study, it became all the more important to learn as much as possible about the structure of the remaining nucleic acid, especially since its 3′- and 5′-extremities are likely to be involved in the replication of the viral RNA.

It has been shown for a number of genomic RNAs of plant viruses that their 3′-termini are folded in a tRNA-like structure which is aminoacylatable by host or heterologous aminoacyl-tRNA synthetases (reviewed in 9–11). They include RNAs from tymoviruses accepting valine, RNAs from bromo-, cucumo-, and hordeiviruses which are tyrosylatable, and RNAs from tobamoviruses, of which TMV is a member, which are histidylatable. It is also known that the 3′-termini of such aminoacylatable viral RNAs have peculiar three-dimensional foldings (*e.g.* 12–15), but which incorporate the essential identity features of canonical tRNAs for discrimination and charging by the relevant synthetases (16, 17). Beside functional arguments, these conclusions are based on structural studies performed on the tRNA-like domains using enzymatic and chemical probing combined with modeling techniques. The 3′-terminus of TMV RNA has tRNA-like properties, first illustrated by its histidylation ability (18). Given the partial sequence homologies between STMV and TMV RNAs, we examined the STMV RNA sequence for a putative tRNA-like secondary folding at its 3′-terminus.

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We propose here the secondary structure for the 3'-end of STMV RNA which presents tRNA-like features. This folding is derived from the knowledge of the tRNA-like folding of tobamovirus RNAs [(19, 20) and Felden *et al.*, in preparation] and of phylogenic comparisons of these RNAs with STMV RNA. The presence of the major histidine identity determinant (21, 22) within the proposed STMV tRNA-like folding suggests that this RNA could be aminoacylated with histidine, as its TMV helper virus. This prediction has been verified and consequently validates the reality of the tRNA-like folding at the very 3'-end of the STMV RNA.

MATERIALS AND METHODS

Enzymes and chemicals

Yeast histidyl-tRNA synthetase (HisRS) was an enzyme preparation enriched by chromatographies on DEAE-cellulose, hydroxyapatite, and phosphocellulose (23). Bacteriophage T7 RNA polymerase was isolated according to (24). Restriction enzyme EcoT22I was purchased from United States Biochemical Corporation (Cleveland, OH, USA). L-[^{3}H] histidine (58 Ci/mmol) was from Amersham (Les Ulis, France). Nucleotides were from Boehringer-Mannheim (Meylan, France). Rotiphorese Gel 40 solutions of acrylamide and N, N'-methylene-bis-acrylamide were from Carl Roth GmbH (Karlsruhe, Germany).

Preparation of STMV and TMV viral RNAs

STMV was purified from *Nicotiana tabacum* leaves co-infected with both TMV U5 strain and STMV essentially as described in (3). TMV virus (Strasbourg strain S) was a kind gift of J.Witz (Strasbourg) and was prepared as described in (25). Both viral RNAs were extracted from their capsids by two phenol extractions and recovered by ethanol precipitation. Spectrophotometric measurements were made to determine viral RNA concentrations, assuming that 1 absorbance unit at 260 nm corresponds to 40 $\mu g.ml^{-1}$ RNA in a 1 cm path-length cell. One mole of STMV RNA and of TMV RNA correspond respectively to 0.3 10^6 and 2.1 10^6 daltons.

Preparation of tRNA^{His} transcript

The plasmid containing the gene of yeast tRNA^{His} was constructed as described (26) and linearized by EcoT22I restriction nuclease before *in vitro* transcription. The transcription mixture contained 40 mM Tris−HCl (pH 8.0), 22 mM $MgCl_2$, 1 mM spermidine, 5 mM dithioerythritol, 0.01% Triton X100, 4 mM each of ATP, CTP, GTP, UTP, 15 μg of linearized DNA for 50 μl transcription mixture, and 495 units of T7 RNA polymerase. After an incubation step of three hours at 37°C, the reaction was stopped by phenol / ether extraction followed by ethanol precipitation. The transcript was separated from non-incorporated nucleotides and DNA fragments by 12% polyacrylamide gel electrophoresis followed by electroelution. This made it possible to separate transcripts ending with the correct 3'-sequence from those presenting one or two additional nucleotides due to T7 RNA polymerase errors during the transcription. Purified transcripts were recovered by ethanol precipitation. Spectrophotometric measurements were made to determine tRNA^{His} concentration as described previously.

Aminoacylation assays

Aminoacylation reactions were performed with full length viral RNAs [1059 nt for STMV RNA (5) and 6395 nt for TMV RNA (27)]. Control aminoacylation was done using a tRNA^{His} transcript, deprived of modified bases, as are the two viral RNAs. Assays were performed at 30°C ina medium containing 25 mM Tris−HCl (pH 7.8), 15 mM $MgCl_2$, 7.5 mM ATP, 50 μM L-[^{3}H] histidine (58 Ci/mmol) with the required concentrations of RNA and yeast histidyl-tRNA synthetase. Aliquots were spotted onto 3MM Whatman papers at different times and trichloroacetic acid precipitated. Kinetic parameters (K_m and V_{max}) were determined from Lineweaver−Burk plots. They represent an average of three independent experiments.

RESULTS AND DISCUSSION

Secondary structure prediction for the 3'-end of the STMV RNA

The secondary structure proposed for the 3'-non coding region of STMV RNA (188 nt) is displayed in Figure 1. It was constructed by mimicking the fold of the homologous region of TMV RNA (20) and by taking into account phylogenetic relationships between RNAs from the tobamoviridae family. In TMV RNA, this region encompasses a tRNA-like fold (19). Within the 188 nt of the STMV tRNA-like fold, 114 (61%) are identical with those of TMV RNA and 48 nt (25%) correspond to 24 compensatory base-pair changes (circled in Fig. 1) distributed in 6 out of the 7 domains of the molecule (only domain E is strictly conserved in sequence and folding in the two RNAs). The remaining 26 nt (14%) correspond to insertions and base changes. Furthermore, two nucleotides are deleted. Interestingly, insertions and deletions concern only single-stranded domains and compensatory changes maintain the double-stranded regions consisting in helices and pseudoknots.

The strict sequence conservation in the 899−949 region of STMV RNA and the 6259−6300 region of TMV U1 RNA (5, 27) (domain E in Figure 1) explains that the stretch of pseudoknots within STMV RNA, as in TMV RNA (20), was easily detected [(28 and this work]. In this region of STMV RNA, no deletion and only a few sequence insertions occur, so that finding of compensatory base changes around conserved core E was facilitated. As already shown for TMV RNA one decade ago (19), a possible tRNA-like folding at the very 3'-end of STMV RNA has also been suggested, although no explicit proposals for a secondary structure were given (10, 29). In fact sequence comparison of the two viral RNAs was not straigthforward, because the presence of deletions and insertions rendered finding of compensatory base changes difficult. Moreover, attempts to define the actual secondary structure of the 3'-terminus of STMV RNA failed because the algorithms used could not predict pseudoknots (7).

Structural arguments supporting the folding of the 3'-end of STMV RNA

Based on the secondary structure established in 1984 by Rietveld *et al.* (19) and on the recent computer modeling of the TMV tRNA-like domain (Felden *et al.*, in preparation) it became possible to understand conformational features of STMV RNA. This is in particular the case for the three-way junction between domains A, B and D that imposes the orientations of the three super domains A, B-C, and D-E-F-G, as well as for the tRNA-like mimicry. Three nucleotides (G59, C92, and C93) are inserted in this three-way junction. They may decrease the structural constraints within the STMV tRNA-like fold and likely lead to an increased flexibility of this fold. Pseudoknot D (formed by

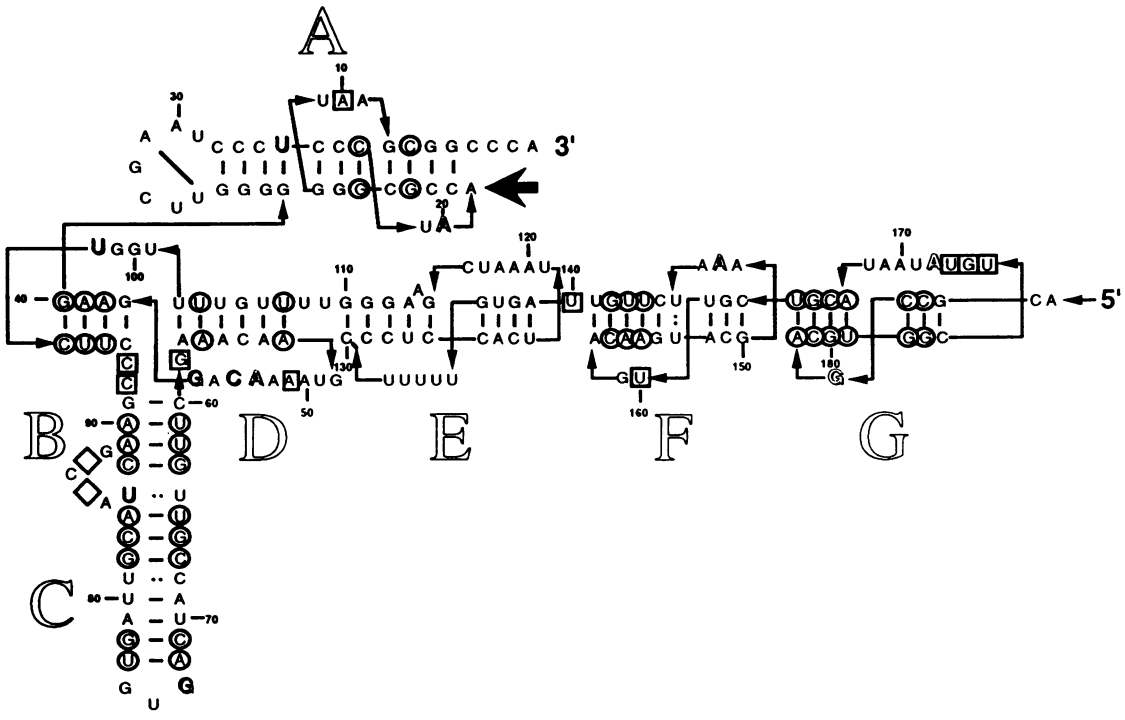

Figure 1. Proposition of a model of secondary structure for the 188 nucleotides located in the 3'-non coding region of STMV RNA based on its primary sequence (5). The secondary structure is proposed by analogy to the folding determined for the 3'-end of TMV RNA (19). Shadowed nucleotides (large lettering) correspond to single sequence changes. Compensatory base changes in helices are highlighted by circles. Diamonds show deletions and squares insertions. All other nucleotides are strictly common to STMV and TMV RNAs. Major structural domains are classified by a letter code ranging from A to G. Non-canonical base-pairs in super domain B-C and in domain F are indicated. The bold arrow emphasizes nucleotide A19 mimicking minus one nucleotide in canonical $tRNA^{His}$. Notice that the T-like loop and pseudoknot E are perfectly conserved in sequence and folding. In contrast to convention, numbering starts at the 3'-end of the molecule. Thus, nucleotide 120 from this representation corresponds to nucleotide 940 in the genomic STMV RNA (5) and to nucleotide 6279 in the genomic TMV RNA (27). Watson–Crick pairing between C92 or C93 and G59 is possible but has not been emphasized in the absence of experimental proves.

nt G40 to A58 and nt C94 to U107), belonging to the three-way junction, show four sequence changes and one insertion within the two single stranded regions (nt G44 to G52 and nt U98 to U101) whereas 10 compensatory base changes in this domain allow conservation of the two stacked helices (see Fig. 1).

Summarizing, the highest conservations between the 3'-terminal sequences of STMV and TMV RNAs concern the helical and pseudoknotted regions; in contrast, the major differences are located in the single-stranded connecting regions. Here, sequence alterations are less important and do not disturb the scaffolding of the molecule. Concerning individual domains, we observe that domain A is similar to the amino acid accepting branch of TMV RNA. It contains a continuous helix made of 11 base-pairs and a seven nucleotide long loop, including a UUCG sequence mimicking the TΨCG loop of canonical tRNAs and a likely A31–U35 Hogsteen pair. Sequence differences within the helix are such that two compensatory base changes restore its expected length. Both loops crossing the deep and shallow groves of the pseudoknot present one additional nucleotide and one sequence change as compared to TMV RNA. Super domain B-C (nt C60 to G91), homologous to the anticodon branch of TMV RNA, contains a 13 base-pair stack closed by a GUG loop corresponding to an histidine anticodon triplet. The helical part of the branch comprises two non-canonical base-pairs (U64–U85 and C68–U81) and an internal bulge (nt A85 to G87) which is two nucleotides shorter than that present in TMV RNA. Taken together, we think that super domain B-C is less stable

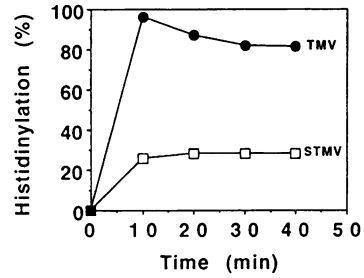

Figure 2. Charging kinetics of STMV (r) and TMV (l) RNAs aminoacylated with yeast HisRS. Experiments were done under optimal enzyme concentration and in the presence of equivalent molar amounts of RNA.

in STMV than in TMV RNA. Furthermore its junction to domain A via pseudoknotted domain D appears less compact due to the likely insertion of the unpaired nucleotides G59, C92, and C93. The resulting increased flexibility may account for the functional differences between STMV and TMV RNAs (see below). Finally, the tRNA-like domain of STMV RNA is followed by a stretch of pseudoknots (E, F, and G). Such an arrangement was already proposed for STMV RNA, but with a slightly different architecture for pseudoknot G (28). The new fold proposed here mimics better the homologous structure present in TMV RNA (20).

Table 1. Kinetic parameters of histidylation of the STMV RNA relative to TMV RNA and to $tRNA^{His}$ transcript

RNA	aminoacylation+ plateaus (%)	K_m (nM)	V_{max} (arbitrary units)	V_{max}/K_m (arbitrary units)	L (relative to TMV-RNA)	L' (relative to $tRNA^{His}$)
STMV RNA	30	650	36	0.055	3.6	17
TMV RNA	98	90	18	0.2	1	4.7
yeast $tRNA^{His}$	100	75	70	0.933	–	1

+Aminoacylation levels were obtained with equivalent amounts of enzyme and correspond to optimal plateaus.
L and L' values correspond to losses of specificity and are expressed by either $(V_{max}/K_m)_{TMV\ RNA}/(V_{max}/K_m)_{STMV\ RNA}$ or $(V_{max}/K_m)_{tRNA^{His}}/(V_{max}/K_m)_{STMV}$ or $_{TMV\ RNA}$ ratios.

Functional predictions

Two functional predictions can be proposed on the basis of the 3'-terminal secondary structure model of STMV RNA. The first one concerns the regulatory mechanism of STMV genome translation. Indeed, it has been recently demonstrated by extended mutational analysis that the pseudoknotted domain in the helper TMV RNA corresponding to the E-F-G region in STMV RNA is directly involved in translational regulation during the TMV life cycle (28). Strong effects occur after single sequence changes in the two pseudoknots homologous to regions E and F in STMV RNA. Interestingly, compensatory mutations restoring these pseudoknots, only restore activity if they occur in the pseudoknot homologous to F, thus indicating the strict sequence requirement of domain E for translational regulation. Because of the strong structural homology between STMV and TMV RNAs in this region (with strict sequence and folding conservation in pseudoknot E, see Fig. 1), it appears likely that both RNAs use the same regulation mechanism for translation of their genomes, especially since STMV is functionally dependent upon the presence of its TMV helper.

Concerning a possible tRNA-like function of STMV RNA, our results predict that this molecule should be charged by histidine. As a prerequisite, it contains at its 3'-end the amino acid accepting $-CCA_{OH}$ sequence universally conserved in amino acid accepting RNAs and a GUG histidine anticodon located in super domain B-C mimicking a tRNA anticodon branch. The presence of this anticodon triplet within STMV RNA was already noticed by Mans *et al.* (10), who thus perceived a tRNA-like potential in this viral RNA. A possible tRNA-like fold at the 3'-end of STMV RNA was also discussed by Solis and Garcia-Arenal when comparing its sequence to that of another tobamovirus RNA, namely tobacco mild green mosaic virus (TMGMV) (29). As TMV RNA, which is histidine specific (18) and also canonical histidine specific tRNAs, STMV RNA contains the major histidine identity element (21, 22) properly located in its structure. This element is a nucleotide at position −1 and is mimicked in STMV RNA by residue A19 in the loop crossing the deep groove of pseudoknot A.

Histidylation of STMV RNA

Figure 2 demonstrates explicitly the histidylation ability of STMV RNA. Using yeast HisRS, up to 30% of the input viral RNA molecules can be histidylated after 10 minutes of incubation with the enzyme. This charging level is high but lower than that found under the same experimental conditions for TMV RNA which can be charged to nearly 100% (the slight decrease in the plateau levels being reproducible).

A more quantitative estimate of the histidylation properties of STMV RNA is given in Table 1. Here we compare the Michaelis–Menten parameters V_{max} and K_m of histidylation of STMV RNA, TMV RNA and yeast $tRNA^{His}$ transcript (we recall that these three molecules are deprived of modified bases and that the viral RNAs are full length molecules). First of all, it can be seen that the histidylation efficiency of STMV RNA is intrinsically high as compared to that of canonical $tRNA^{His}$, the usual substrate of yeast HisRS; in terms of relative catalytic efficiency it is only 17-fold less active than $tRNA^{His}$ transcript (thus TMV RNA charging is about 5-fold less efficient than $tRNA^{His}$ charging). The main outcome of this work, however, is that the catalytic efficiency of the histidylation of STMV RNA is quasi-equivalent to that of TMV RNA (as expressed in relative V_{max}/K_m values). Indeed, STMV RNA charging is only 3.6-fold less efficient than TMV RNA. Interestingly, however, the two molecules behave differently with HisRS since the K_m for STMV RNA is significantly higher than that for TMV RNA (650 *vs* 90 nM) suggesting a lower affinity of the satellite RNA for HisRS. This detrimental effect is partly overcome by a two-fold higher V_{max} for charging of the STMV RNA. The reasons for these mechanistic differences are not clearly understood, but it may be proposed that a greater flexibility of STMV RNA (because of the presence of non-canonical base-pairs and of insertions, see above) could weaken affinity with HisRS (K_m effect) but in contrast also facilitate the functional adaptation of the RNA with the synthetase (V_{max} effect).

The kinetic differences between STMV and TMV RNA charging could also be due to the presence of different levels of unchargeable molecules in the two RNA preparations. The fact that the aminoacylation plateau of STMV is lower than that of TMV RNA (see Fig. 2) agrees with this view. Inactive molecules could arise from the greater structural plasticity of STMV RNA which would favour alternate foldings of the molecule. An attractive possibility would be a circularization of the RNA by pairing of its 5'- and 3'-termini. Such circularization has already been discussed for plant viral RNAs terminating with a tRNA-like domain (30) and its occurrence is also possible for STMV RNA (7).

General conclusions and biological perspectives

RNA viruses are often accompanied by satellite viruses as found in many instances with plant viruses. Satellite and helper viruses are functionally related since they infect the same host and since replication of the satellite is dependent upon that of its helper (31–33). However the mechanisms of this dependence are mostly not well defined at the molecular level. Up to now it was not clear whether this functional relationship occurs also at the structural level, especially since no clear sequence homologies between RNAs from helper and satellite viruses exist (32). Exceptions concern the virulent satellite RNA of turnip crinkle

virus (34) and to a lesser extent the satellite RNA of cucumber mosaic virus (35), in which some similarity with the genomic helper RNAs appears at the 3'-termini. We report here a strong structural homology at the level of the secondary and tertiary structure between the 3'-termini of the RNAs from STMV and TMV.

TMV RNA presents an aminoacylatable tRNA-like domain at its 3'-end as do several other plant viral genomic RNAs [reviewed in (9–11)]. These domains are not involved in protein synthesis (9, 36) and no general rule has so far appeared for their occurrence and for the necessity of their aminoacylation. As a general role of these structures, a tempting suggestion is that of a telomer function which would protect the viral RNAs from exoribonuclease cleavages (37–38). Other hypotheses have been proposed (30), but only three have been experimentally documented. In TMV RNA, the tRNA-like domain increases both translational efficiency and RNA stability (39). In BMV RNA the tRNA-like domain is the initiation site for minus strand synthesis, although aminoacylation seems not to be required (40, 41). Finally and in contrast, replication is dependent upon aminoacylation of the tRNA-like domain in TYMV RNA (42).

We note that another virus infecting tobacco, tobacco necrosis virus (TNV) can also be accompanied by a satellite virus (STNV). Although a mimicry with $tRNA^{Met}$ was suggested at the 3'-terminus of STNV RNA (43), it is likely that no tRNA-like folding exists at that level (44). Moreover, no apparent sequence similarity exists between this RNA and its helper RNA (45). This situation contrasts with what observed in TMV and STMV RNAs. It follows that different satellite viruses, even infecting the same host, may require different mechanisms for their expression.

Concluding, we describe for the first time the presence of an aminoacylatable tRNA-like structure at the 3'-end of the RNA from a satellite virus. This tRNA-like structure is the only domain within STMV RNA bearing strong sequence similarity with the helper TMV RNA. This similarity is even higher at the level of the secondary and tertiary structures. Since replication of STMV RNA is strictly dependent upon the presence of its helper, and since both RNAs possess a similar tRNA-like domain at their 3'-termini, it appears likely that this domain represents a recognition signal for the common viral replicase. Thus STMV RNA would use the same replicative mechanism as the RNA from the helper TMV. Following this view, and as shown for TMV RNA (39), we propose that the tRNA-like structure in STMV is essential for translational efficiency and stability of its RNA. This prediction can be verified with engineered STMV mutants lacking their tRNA-like termini which should become inefficient for replication and/or translation.

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